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BIOLOGICAL BULLETIN

IODINE AND AMPHIBIAN METAMORPHOSIS.[1]

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Recently the claim has been made that the metamorphosis of urodele larvæ such as axolotl and amblystoma differs from that of anurans in that transformation in the caudate amphibia is independent of iodine and subject only to the influence of the thyroid gland. Experiments are described in this paper which invalidate this conclusion and indeed, render it impossible to understand such a view. The rôle of iodine in metamorphosis is also discussed, and evidence presented showing that the physiologic response (metabolic changes) of mammals to thyroid administration and the metamorphic changes of amphibian larvæ following iodine treatment are probably due to quite different causes and hence not to be compared.

Acknowledgement is due Mr. William Anderson for his labor in iodizing the various compounds employed.

The axolotls came from New Mexico, and were obtained through the courtesy of Mr. J. N. Gladding of Albuquerque.

I. EXPERIMENTS ON THYROIDECTOMIZED AXOLOTLS.

The thyroid glands of eight animals were extirpated. One larva was extremely large, measuring over a foot in length. The remaining larvæ averaged seven inches total length. Few technical difficulties are involved in removing the thyroid glands from animals as large as the axolotl. The larvæ were anesthetized in chloretone solutions, placed upon their back under a low-power binocular microscope, and the hyobranchial region strongly illuminated. A median incision was made through the skin and superficial muscles, extending from the posterior edge

[1] Part of the expense of this investigation was defrayed by a grant from the Elizabeth Thompson Science Fund.

of the hyobranchial region to the symphysis of the mandible. The skin and superficial muscles were pulled apart on either side and pinned down, exposing the geniohyoid and ceratohyoid muscles. The thyroid glands are located in the triangle on each side of the median line, formed by the geniohyoid and ceratohyoid muscles. The large size of the glands and their intimate relation to the blood vessels which traverse the triangle render their location easy. They can be removed from both sides of the animal with but little injury to the blood vessels if first dissected free with a razor-edge needle and gently pulled out with a fine-pointed pair of forceps. Even if the blood-vessels are injured and considerable bleeding occurs, the post-operative effects upon the animal are negligible. It is surprising how much surgical manipulation an axolotl can withstand without showing any post-operative symptoms. Following gland removal the skin and superficial muscles are sutured along the median line. The animals quickly recover from the anesthetic and appear to suffer no ill effects from the operation. Food was witheld for four or five days after thyroidectomy in order to prevent any possibility of tearing the sutures while swallowing.

The thyroidless animals were kept for five months in large concrete tanks through which fresh water ran constantly. The food consisted of worms, insect larvæ, and occasionally, pieces of fresh liver.

Five months following thyroid removal, three animals were injected twice, at five day intervals with eighty milligrams of tyrosine in which two atoms of iodine had been substituted for two hydrogen atoms of the molecule, forming the well-known compound 3–5–diiodotyrosine. The animals metamorphosed within seventeen days following the first injection.

Three control thyroidless axolotls injected with equal quantities of pure tyrosine and 3–5 dibromtyrosine, *i.e.*, tyrosine in which two bromine atoms had been substituted for two hydrogens, failed to transform. Later, one of the controls was injected with a third dose of eighty milligrams of dibromtyrosine but with negative results. Dissection of the metamorphosed iodotyrosine-injected animals showed no trace of thyroid tissue present.

Rogoff and Marine ('17) showed that iodized blood serum accelerates metamorphosis of normal tadpoles, and that the

globulin fraction of the serum contained most of the iodine. It was decided to try injecting iodoserumglobulin into thyroidless axolotls, controlling the experiment by injections of equal amounts of non-iodized globulin. The blood of beef was used.

Three axolotls were each injected twice, at eight-day intervals with 110 milligrams of iodoserumglobulin. Metamorphosis resulted within twenty days following the first injection. One of the axolotls had previously served as a control in the iodotyrosine experiment and had been injected with pure tyrosine. The results were negative and as the larva showed no indications of metamorphosis several weeks later it was utilized in the iodoserumglobulin experiment. Two control thyroidless animals (previously used as controls in the iodotyrosine work) injected with large amounts of non-iodized serumglobulin failed to transform.

Dissection of the metamorphosed iodoserumglobulin-injected animals showed two of them to have no vestige of thyroid tissue, but the remaining one had a portion of the gland present on the left side, an amount about equal to a third of the entire thyroid. One of the controls was then injected with iodoserumglobulin and metamorphosed twenty-one days after the first injection; dissection of this animal also showed a small nodule of thyroid tissue present. Thus four axolotls were metamorphosed by injections of iodized serumglobulin; two of the animals were completely thyroidectomized, and two only partially. No very marked difference in the time or rate of metamorphosis was noted between the partially and completely thyroidectomized animals. The axolotls possessing remnants of glands were the first to show signs of transformation following injection, but the time difference was not over three days in any case. The fact that both types of animals metamorphosed within a few days of each other may have been due to the large amount of iodoserumglobulin injected. It is probable that partially thyroidectomized animals will be found to respond by metamorphosis to considerably smaller doses than thyroidless animals. Axolotls were not available to test this point.

Considerable difficulty was experienced injecting the globulin intraperitoneally. The solution of the problem while crude was effective. A small incision was made through the ventral body wall sufficiently large to admit the end of a graduated pipette

and the globulin was injected in powdered form. This method because of the ease with which it could be performed was also utilized in the tyrosine experiments. Forced feeding of the animals with the various substances by means of a pipette thrust down the œsophagus into the stomach, might have been as effective as injections, but the animals usually regurgitate some of the material, at any rate this was found to be the case in an earlier experiment where desiccated thyroid tissue was administered by forced feeding.

The pigment pattern characteristic of adult Amblystoma tigrinum is not completely developed in axolotls until several weeks following metamorphosis.

Jensen ('21) metamorphosed axolotls (with intact thyroid apparatus) by injections of iodized casein, iodoserumglobulin, and iodoserumalbumin. He also removed the thyroid glands of axolotls and attempted to metamorphose the larvæ by injections of iodized proteins but his animals died. He concluded that such iodized proteins are highly toxic for thyroidless animals but not for those possessing normal glands. In my own experiments the axolotls withstood the injections of iodized amino-acid and serumglobulin as well as those with pieces of the gland present. There was nothing in the behavior of the animals to indicate that iodized substances are more toxic for thyroidless axolotls than for normal animals. Iodized substances are certainly not more toxic for thyroidless anuran tadpoles than for normal larvæ.

In an earlier experiment than those recorded here three axolotls were thyroidectomized and injected with large doses of iodotyrosine. The animals were kept in ordinary glass aquaria without running water. All of the animals died within ten days following injection. In later experiments the injected axolotls were kept in large concrete tanks, filled to capacity and with fresh water running constantly. None of the animals died. Intraperitoneal injections of large doses of either iodotyrosine or iodoserumglobulin has a marked depressing effect upon thyroidless axolotls; the animals are sluggish, move about very little, refuse food, and show indications of weakness for several days or even a week following injection.[2]

[2] A large axolotl was thyroidectomized and kept for eight months then twice injected at eight day intervals with large amounts of iodized casein. Metamorphosis resulted within twenty-nine days.

The experiment shows beyond doubt that thyroidless urodele larvæ respond to iodized substances in precisely the same manner as thyroidless and pituitaryless anurans. The importance of iodine in the amino-acid and protein molecule in order to render these substances effective in inducing metamorphosis is clearly demonstrated.

II. Experiments on Larval *Spelerpes bislineatus*.

H. H. Wilder years ago ('99) called attention to *Spelerpes* as a favorable object for experimentation. Unlike most urodeles, the larvæ of *Spelerpes* can be obtained at any season of the year and are easily kept under laboratory conditions. The larval life of this form has not been adequately investigated, consequently little is known concerning it. I. W. Wilder ('22) has been engaged for some years in studying the relation of growth to metamorphosis but to date has published only a very brief summary of the results. The data indicate a large range of variation in the size and age of the animals at transformation, with an average larval life of two years.

Eighty animals were used in the experiment, varying in size from 23 mm. to 53 mm. total length. They were separated into four groups of twenty animals each, equal numbers of large and small larvæ being represented. One group of twenty animals was kept in a large aquarium and given plenty of food. The three remaining cultures were subdivided into smaller lots of ten animals each and kept in glass containers in 250 cc. of tap water water through which compressed air bubbled constantly. *Spelerpes* larvæ soon die if kept in small amounts of water unless it is kept cool and well ærated. Twenty individuals were reared in tyrosine solutions representing 120 mg. per 250 cc. of water; another twenty were kept in equivalent solutions of 3-5 dibromtyrosine; twenty more in equal concentrations of 3-5 diiodotyrosine. The animals were fed sparingly during the experiment. Once each week they were removed from the tyrosine solutions and placed in large containers holding 6,000 cc. of water, plentifully supplied with food and allowed to feed for thirty-six hours after which they were returned to the solutions.

The experiment began October 17. Thirteen days from the date the animals were placed in the solutions, two large larvæ of

the iodotyrosine culture showed marked gill and tail-fin reduction. None of the other animals of this or other cultures showed any change. However, by November 5, *i.e.*, twenty days from the first administration of iodotyrosine, all of the larvæ kept in solutions of this substance had metamorphosed. The external gills had disappeared, the gill clefts had closed and the tail fin was completely resorbed. The animals left the water and crawled up the sides of the container.

Examination of the tyrosine and dibromtyrosine cultures showed no indications of metamorphosis. The animals of these cultures were kept in the solutions for a month longer but metamorphosis was not induced. At the close of the experiment exceedingly strong concentrations of tyrosine and dibromtyrosine were employed but with negative results. The larvæ of the normal culture kept in the large aquarium and plentifully supplied with food likewise failed to metamorphose or to show any indications of transformation a month after the animals of the iodotyrosine culture had completed the process.

The results of this experiment are quite clean cut and admit of but one interpretation: It is the iodine within the tyrosine molecule that is responsible for the induced metamorphosis because the tyrosine and dibromtyrosine were ineffective either in weak or strong concentrations when administered over comparatively long periods.

Efforts were made to thyroidectomize the larvæ but without success owing to the extremely small size of the thyroid glands. The thyroid apparatus of *Spelerpes* larvæ of 47 mm. total length does not contain enough of the physiologically active hormone to induce metamorphosis in anuran tadpoles when heteroplastically transplanted. This experiment was attempted by Mr. O. M. Helff of this laboratory but without success.

Judging by the positive results obtained with thyroidless axolotls, it is highly probable that thyroidless *Spelerpes* would react by rapid metamorphosis if injected with, or reared in solutions of iodotyrosine. It is interesting to note that both large and small larvæ metamorphosed within twenty days, though the differences in size were great, varying as they did from 23 mm. to 52 mm. total length. Probably some of the smaller animals were considerably younger than the larger ones, however, it is

impossible to make any definite statements on the point until more is known about the relation of size to age in the larvæ of this urodele.

The fact is well known that the thyroid gland of vertebrates exhibits a remarkable selective action in regard to iodine absorption, taking this element from the blood and synthesizing it into the thyroid hormone by the addition of other substances. In view of this property of thyroid tissue, it is possible, though rather improbable, that in the experiment just cited, the thyroid apparatus of the iodotyrosine-fed animals took up the iodine pouring into the organism and elaborated excessive quantities of the hormone, thus inducing metamorphosis. However, the rapid transformation of both thyroidless frog and salamander larvæ when fed or injected with iodized proteins and amino acids renders such an assumption doubtful.

It is interesting to note that Huxley and Hogben metamorphosed *Salamandra* and *Triton* larvæ by rearing them in dilute solutions of inorganic iodine. But in these experiments the thyroid glands of the animals were intact, hence it is impossible to know whether the action of the iodine was direct or through the mediation of the thyroid.

III. Experiments on *Amblystoma punctatum*.

It was considered desirable to test the effects of iodo- and bromtyrosine upon the metamorphosis of *Amblystoma* since it was owing to negative results obtained by administration of inorganic iodine to animals of this group that led Uhlenhuth to assert that iodine has no effect upon salamander transformation.

Eighty young larvæ of *Amblystoma punctatum*, averaging 30 mm. total length were divided into four groups of twenty animals each. The larvæ of three groups were isolated in finger bowls containing 50 c.c of water, one animal to each container. The twenty larvæ remaining were reared in large aquaria and fed quantities of tubifex. The animals in the finger bowls received food only at definite intervals and in very small amounts so that they were in a state of semi-starvation during the course of the experiment.

Twenty larvæ received small amounts of diodotyrosine crystals dissolved in the 50 cc. of water in the finger bowls. Twenty

larvæ were fed equal quantities of dibromtyrosine crystals, whereas the remaining twenty animals received no food of any kind and served as controls. Such a culture was considered necessary in order to note any effects starvation might bring about on the progress of metamorphosis since the animals reared on the tyrosine compounds were given very little food.

It should be pointed out here that the animals in the dibromtyrosine solutions received considerably more bromine than the animals of the iodotyrosine cultures received iodine, despite the fact that equal quantities of the two substances were fed to each larva. This is obvious enough since the atomic weight of iodine is 126.92, while that of bromine is but 79.92. Consequently if equal amounts of the two tyrosine compounds were fed or put into solution in given amounts of water as was the case in the present experiment, the number of bromine atoms per milligram of dibromtyrosine would be nearly double the number of iodine atoms per milligram of diiodotyrosine. In all the experiments recorded in this paper this fact has been ignored and the two tyrosine compounds have been administered in equal amounts as though they were chemically equivalent. The experiment began June 22, 1923. On this date none of the small immature larvæ revealed the slightest indication of metamorphosis. June 28 all animals of the iodotyrosine-fed culture showed marked reduction of the gills—three animals had only the stumps remaining. The tail-fin was undergoing reduction. Sand was placed in each finger bowl in order that the animals might crawl out of the water as metamorphosis progressed; this is a necessary precaution, otherwise the animals will drown over night.

Examination of the larvæ reared in the dibromtyrosine solution showed no change; this was also true of the fed and unfed control cultures.

July 4, twelve days from the beginning of the experiment, all of the diiodotyrosine-fed larvæ had completely transformed and left the water. Their gills and tail-fin had disappeared; the branchial clefts had closed and the larvæ had shed their skins but the pigmentation characteristic of the adults of this species had not appeared. Fig. 1, *A*, is a photograph of four iodotyrosine-fed animals. Compare this with Fig. 1, *B*, which

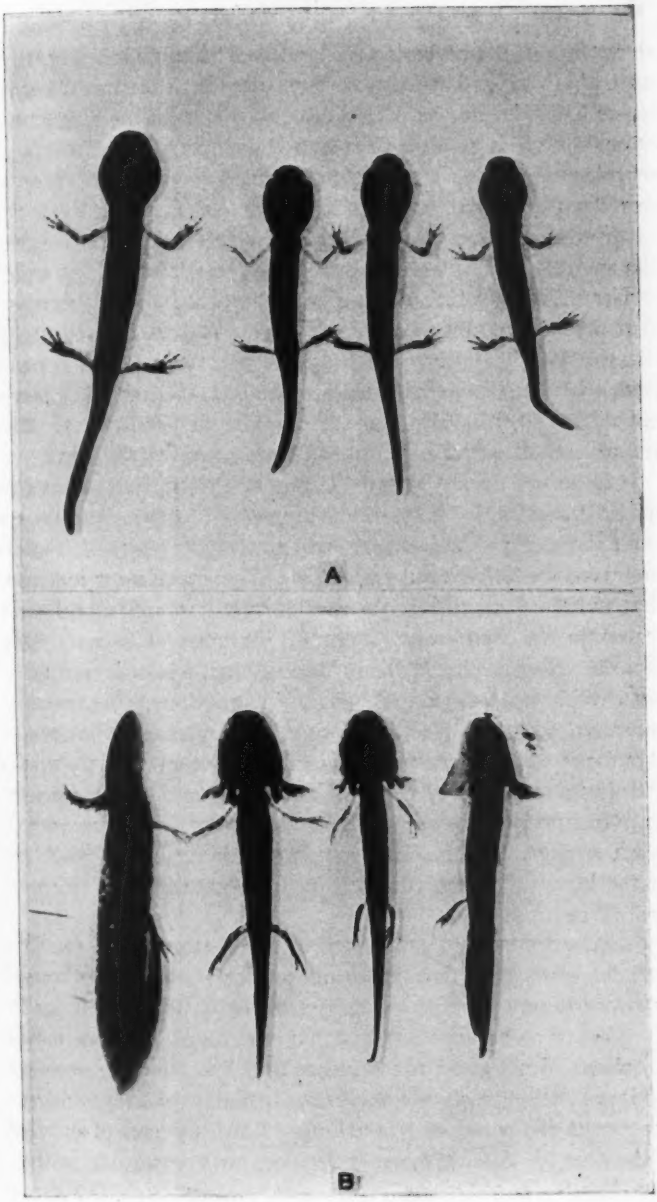

Fig. 1. *A*. Precocious metamorphosis of immature *Amblystoma* larvæ after twelve days immersion in iodotyrosine solution. × 2. *B*. Immature larvæ of *Amblystoma* after twenty-five days immersion in dibromtyrosine solution. × 2.

shows four di-bromtyrosine-fed larvæ. The animals in Fig. 1, *B*, were photographed twenty-five days after the experiment began, hence had been on the dibromtyrosine diet twice as long as the animals of Fig. 1, *A*, had been fed iodotyrosine. The metamorphosed animals were very weak and died within three to five days after metamorphosis. During the twelve days the experiment continued, they were each fed three small worms, the animals of the other cultures (except one) received the same.

The dibromtyrosine culture was continued twenty-six days after transformation of the iodotyrosine culture had occurred, *i.e.*, thirty-eight days from the date of first feeding. At the end of this time eleven animals metamorphosed, the remaining larvæ did not transform. The culture was abandoned July 31. The fed and unfed control groups were also given up at this time.

It is an interesting fact that none of the control animals of either starved or fed cultures metamorphosed during the thirty-eight days of the experiment, whereas eleven larvæ of the dibromtyrosine culture did transform. The experiment indicates that unfed salamander larvæ can be forced to metamorphose if reared in very strong solutions of dibromtyrosine over long periods. The bromine in the tyrosine molecule is thus seen to be not entirely inert as regards metamorphosis though it cannot be compared with iodine. For instance, diiodotyrosine solutions of about one-half the concentration of the dibromtyrosine, metamorphosed salamander larvæ of equal size and developmental stage in a period ranging from seven to twelve days, whereas the much stronger bromtyrosine solutions caused about two-thirds of the larvæ of the culture to transform between the thirtieth and thirty-ninth day.

In an earlier paper ('22) the writer called attention to the fact that the hind legs of thyroidless and pituitaryless tadpoles reared in strong dibromtyrosine solutions grow larger than the limbs of like animals on an algæ diet but that such animals do not metamorphose. Here again the evidence indicates that the bromine ion is not entirely passive since it does stimulate to a slight degree the growth of the tadpole's hind limbs. But the degree of activity of the bromine in the tyrosine molecule is not comparable to that of iodine. The following experiment clearly shows the great difference in activity between the two substances. Three sets of

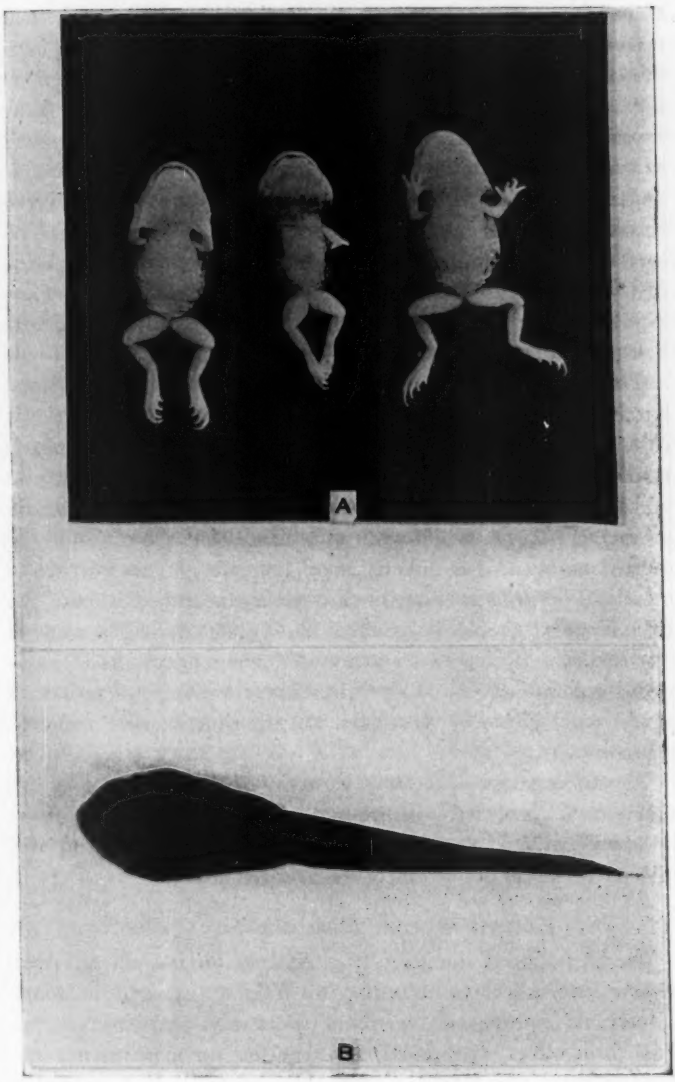

Fig. 2. *A*. Immature *Rana clamitans* larva metamorphosed by immersion in iodotyrosine solution for fifteen days. *B*. *Rana clamitans* tadpole reared for twenty days in dibromtyrosine solution.

thyroidless and pituitaryless *R. sylvatica* larvæ were used. One set of animals was reared in tyrosine solution, another in iodotyrosine, a third set in dibromtyrosine of twice the strength of the iodotyrosine solution. No food of any kind was given. The iodotyrosine cluture metamorphosed within fifteen days, the animals of tyrosine and dibromtyrosine culture showed no indications of metamorphosis after forty-three days. However, the larvæ reared in the dibromtyrosine solutions had well developed hind legs averaging 7 mm. total length as compared with an average of 3.5 for the animals of the tyrosine culture. Since no food of any kind was given the increase in limb growth was probably due to a slight influence exerted by the bromine ion.

The result of this experiment upon *Amblystoma punctatum* larvæ is identical with that obtained with *Spelerpes*, and shows clearly that it is the iodine in the tyrosine molecule that is responsible for the precocious metamorphosis. The rapidity with which the metamorphic response to iodotyrosine is evoked in salamander larvæ, would seem to indicate that the action of the iodized amino-acid is directly upon the cells and tissues of the organism and not necessarily through the intermediation of the thyroid gland. It will be recalled, that young thyroidless axolotls (*Amblystoma tigrinum*) transform following injections of iodotyrosine and it is probable that thyroidless *Amblystoma punctatum* would react likewise. However, no experiments were made to test this point.

The writer now has under way a series of experiments upon the metamorphosis of thyroidectomized salamander and anuran larvæ in which various iodized proteins and amino acids are fed and injected. The results will be communicated later.

IV. Experiments on *Rana clamitans* Tadpoles.

The larval life of the green frog, *Rana clamitans*, extends over a year (370–400 days according to Wright, '14) and in some individuals is prolonged two years before metamorphosis occurs. This form offers exceptional opportunities for experimentation because of the long duration of larval existence, and is especially valuable for use in investigations on metamorphosis where the feeding method is employed. Tadpoles of all sizes are obtainable at any season of the year and when brought to the laboratory

readily adapt themselves to the changed environment. Animals captured in the autumn generally pass the winter and spring as tadpoles.

September 13, 1922, a large number of larvæ were collected and eighty tadpoles of approximately equal size and developmental stage were selected and divided into four groups of twenty individuals each. One group was fed tyrosine, another ordinary tadpole food such as spirogyra and insect larvæ, the third lot received dibromtyrosine and the fourth group diiodotyrosine. A fifth culture of animals, forty in number, of varying size and developmental stage was kept in large glass aquaria and fed quantities of algæ. Each culture except the fifth was further subdivided into lots of five tadpoles each, and placed in 250 cc. of tap water containing 120 mg. of the tyrosine compounds. Each evening the animals were transferred to large jars containing 10,000 cc. of tap water and plentifully supplied with algæ. This procedure was considered necessary in order to rule out the starvation factor since tadpoles can not exist indefinitely on tyrosine alone.

The animals obtained the tyrosine compounds by two methods: through the alimentary tract, and by absorption through the skin. Most of the tyrosine goes into solution after a short time, hence the larvæ probably obtain most of this substance through the skin. This is certainly the manner in which *Spelerpes* larvæ obtain the tyrosine and its iodized and brominated compounds.

Table I. gives the average measurements of the animals at the beginning and end of the experiment. The figures are averages based upon measurements of fifteen animals of each culture.

TABLE I.

	Algæ-Fed.		Tyrosine.		Dibromtyrosine.		Diiodotyrosine.	
	Total Length, mm.	Hind Legs, mm.	Total Length, mm.	Hind Legs, mm.	Total Length, mm.	Hind Legs, mm.	Total Length, mm.	Hind Legs, mm.
Sept. 13.....	51.2	3.9	52	3.5	52.2	3.8	51.5	3.5
Oct. 8.....	52.2	3.9	52.5	3.8	52.5	3.9	Advanced stages of metamorphosis	

Thirteen days from the beginning of the experiment a marked

difference was observed between the iodotyrosine-fed animals and those of the other cultures. The tyrosine and dibromtyrosine-fed tadpoles showed no changes either in regard to growth or metamorphosis from the algæ-fed controls, whereas the iodotyrosine-fed animals appeared thin and emaciated; the hind legs had increased in length also the skin of the pectoral region where later the fore-legs appear, was undergoing autolysis.

October 3, four of the iodotyrosine-fed tadpoles had fore legs and frog mouths. The hind legs of all of the animals had markedly increased in length, and the tail was undergoing resorption. In several individuals large patches of skin over the region of the fore-limbs was totally destroyed by autolysis. The animals of the remaining cultures showed no change.

October 6, the animals of the iodotyrosine culture were all in advanced stages of metamorphosis and many were dying; only one animal remained alive on October 8. Table I. gives the measurements of the tadpoles of the various cultures at the close of the diiodotyrosine experiment, twenty-three days after the date of first feeding.

The remaining cultures were continued until October 30. The tyrosine and dibromtyrosine-fed tadpoles were then taken off the tyrosine diet and fed algæ. The larvæ were measured November 13 two months after the beginning of the experiment but no indications of metamorphic change was observed. It is quite evident that insofar as the metamorphosis of the green frog is concerned, tyrosine and dibromtyrosine are ineffective, even when administered in large quantities. These results are in agreement with those obtained in similar experiments on thyroidless axolotls and thyroidless and pituitaryless anuran tadpoles (Swingle, '22).

Discussion.

The experiments described in this paper and elsewhere on the administration of iodine, iodized amino-acids and proteins to thyroidless and pituitaryless anuran larvæ and to thyroidless axolotls demonstrate that other forms of iodine than that peculiar to the thyroid hormone (thyroid iodine) possess the power of inducing amphibian transformation. This property of iodine is apparently unique, since so far as known at present it is not shared by other substances, and is inherent in the iodine atom when

organically combined in a certain way. The type of combination is not necessarily that characteristic of thyroid iodine because a large number of iodine compounds have the power to bring on metamorphosis in thyroidless amphibians—even elemental iodine itself. That elemental iodine is utilized within the organism as iodine per se for any purpose seems improbable because if one compares the effect of administering inorganic iodine and various organic preparations to thyroidless tadpoles, it becomes evident that the physiologic activity and metamorphosis-inducing properties of the organic preparations are superior to elemental iodine. Furthemore, the accelerating effect upon metamorphosis of the organic iodine compounds is less than that of the thyroid extract itself. However, the important thing is not the speed with which iodine compounds induce metamorphosis in comparison with the thyroid hormone itself, but the fact that iodine other than thyroid iodine induces the metamorphosis of thyroidless animals. The crux of the problem in regard to amphibian metamorphosis, is to find out just what it is in tyrosine, serumalbumen, serumglobulin, casein, tyramine and probably a host of other amino acids and proteins which when iodine is added increases so remarkably the metamorphosis-inducing powers of this element. Why, for example, does the linking of iodine to the third and fifth carbon atoms of the benzene ring in the tyrosine molecule transform this inert (so far as metamorphosis is concerned) amino acid into a highly active agent. What is it in casein, albumen, globulin or tyrosine that raises so greatly the reactive powers of iodine?

There can be little doubt that elemental iodine when it induces the transformation of thyroidless and pituitaryless tadpoles combines either with the proteins of the algæ fed along with it, or within the body of the tadpole after absorption through the skin or alimentary tract, but it doesn't combine with anything produced by thyroid tissue because there was none present nor had there ever been any present in the case of the thyroidless anurans.

Hirschler ('22) precociously metamorphosed tadpoles by inserting small pieces of elemental iodine into the body of the larvæ. However, since the animals possessed intact thyroid glands their activity can not be ruled out in this experiment,

because the metamorphosis may have been due to increased activity of the thyroid due to increased iodine supply. On the other hand the absorbed iodine may have combined with other than thyroid proteins thus inducing metamorphosis. Either or both of these possibilities may have been realized in Hirschler's experiment, consequently work involving iodine administration should be performed only on thyroidectomized larvæ since only this type of experiment will shed any light upon the rôle of iodine in amphibian metamorphosis. This is especially true of neotenous forms like axolotl; no work on the rôle of iodine in the metamorphosis of this form should be regarded as conclusive unless performed upon animals from which all trace of thryoid tissue has been removed.

Thyroid conditions in axolotl are very peculiar (Swingle, '22) and vitiate the results of experiments done on animals with intact glands. The New Mexican strain of axolotl has perfectly developed thyroids, the vesicles filled to capacity with the physiologically active hormone yet the secretion is apparently unable to escape into the blood stream in sufficient quantities to transform the animal. This is demonstrated by heteroplastic thyroid transplants. An axolotl thyroid is sufficient to metamorphose thirteen normal, thyroidless and pituitaryless anuran tadpoles when grafted, but left intact within the axolot's body is incapable of initiating metamorphosis.[1] It is obvious that in this form we are dealing with a thyroid mechanism which selects, stores and transforms the iodine of the animal's food and water into the thyroid hormone but fails to release the elaborated product. Consequently, if axolotls are fed elemental iodine they do not metamorphose, and for exactly the same reason, they fail to transform under the ordinary dietary regime—the iodine is picked up by the gland and synthesized but not released. It is probable that feeding organic iodine preparations would give similar results. On the other hand it is erroneous to conclude from such an experiment that iodine has no influence on axolotl metamorphosis. Our experiments on both thyroidectomized and partially thyroidectomized axolotls show quite clearly that iodized amino acids and proteins promptly metamorphose these

[1] This experiment was performed by Mr. Karl Mason, of this laboratory, and reported at the Boston meeting of the American Society of Zoölogists, 1922.

animals, and that the amino-acid and protein without the iodine in the molecule are inert.

Hirschler ('22) metamorphosed axolotls (the European strain which rarely spontaneously transforms) by implanting iodoform paste within the body cavity. However, in this experiment the glands were intact so it may be that the iodoform merely served to stimulate the thyroid mechanism to release its stored hormone thus inducing metamorphosis. It will be recalled that Kaufman ('18) metamorphosed axolotl by injections of salicylic acid. So far as is known salicylic acid has no influence on metamorphosis, the effect of injecting the substance into axolotl was to stimulate the secretory activity of the thyroid apparatus in some way. Anyone not familiar with thyroid conditions in axolotl might conclude that salicylic acid per se was the metamorphosis-inducing agent, whereas probably nothing could be farther from the real facts of the case.

The New Mexican strain of axolotl if removed from its native habitat to New Haven soon undergoes spontaneous metamorphosis. Why? Certainly not because the railroad journey exerts any mysterious metamorphosis-inducing power, but probably because the changed food, water, the jolting and confinement incident to the trip acted as a stimulating agent thus releasing the thyroid hormone from the gland vesicles thereby causing transformation.

Where the thyroid apparatus is left intact the fate of the substances fed or injected into an amphibian larva is problematical. If an effect upon metamorphosis is produced it is impossible to determine whether the effect is due directly to the substance itself, or indirectly through intermediation of the thyroid unless the work is checked by repeating it upon thyroidless forms. An excellent illustration of this statement has been furnished by Allen ('20). Allen observed that transplantation of the anterior lobe of the pituitary into hypophysectomized tadpoles (but possessing a rudimentary and functionless thyroid apparatus) metamorphosed the animals, whereas transplantation of the pituitary gland into thyroidectomized larvæ had no such effect. The pituitary secretion had no direct influence upon metamorphosis but acted indirectly by stimulating the rudimentary thyroid into functional activity. Any one working with

normal tadpoles with intact thyroids uncontrolled by thyroidless animals might well have concluded from the results of such an experiment that pituitary tissue exerts a direct stimulus to metamorphosis and is thus equivalent to the thyroid.

Recently ('22) Romeis claims to have isolated an absolutely iodine-free substance from the thyroid gland which when administered to frog tadpoles exhibits all of the physiologic effects of thyroid gland tissue. The writer is skeptical of the validity of this claim and for several reasons: (1) All active substances so far isolated from the thyroid contain large amounts of iodine. Thyroxin the active principle contains sixty-five per cent. of iodine as an integral part of the molecule; and it is known that thyreoglobulin containing no iodine is physiologically inert; (2) Experiments on tadpoles have shown (Rogoff, '18–19) that blood coming from hyperplastic thyroid glands with extremely low iodine content fails to induce tadpole metamorphosis; (3) To date the only substances known to metamorphose thyroidless tadpoles are thyroid or iodine in some form—presumably in the last analysis organically combined. While writing this paper the writer came across a second communication from Romeis which practically amounts to a retraction of his earlier claims. He found that iodothyrine and iodothyroglobulin will induce the metamorphosis of tadpoles in dilutions of one in a million; thyroxin produced the same effects on growth and metamorphosis in dilutions of one in ten million consequently says Romeis: These figures suggest that effects (on tadpoles) from so-called iodine-free materials from the thyroid may have been contaminated with minute amounts of thyroxin.

There can be little doubt that this is the real explanation of the results obtained by this investigator with so-called iodine-free substances from the thyroid.

There seems to exist a fundamental difference between mammals and amphibians in regard to their physiologic response to thyroid and iodine administration. This difference is not sufficiently understood by those who attempt to compare these two verebrate groups. The following experiment of Kendall ('19) is an excellent illustration of the point: He found that injections of pure thyroxin into mammals is followed by a very definite and marked physiologic response. But when the hydrogen of the

imino group in the thyroxin is replaced with acetyl, the substance loses its physiologic activity and there follows no demonstrable effect upon the metabolic rate. This emphasized the importance of the imino group in thyroxin (in so far as the metabolic effect upon mammals is concerned) and minimizes the importance of the iodine in the molecule. Bearing in mind the effect of iodine administration upon tadpole metamorphosis, Kendall was led to try the acetyl derivative of thyroxin on tadpoles, for if metamorphosis depends only upon the increase in the basal metabolic rate of the larvæ then thyroxin should increase the rate of metamorphosis but the acetyl derivative involving the imino group should not. If, however, iodine alone is concerned in accelerating metamorphosis, then both thyroxin and the derivative should affect the transformation. Kendall found that both thyroxin and the acetyl derivative would induce a rapid metamorphosis of the bull frog tadpole. Kendall's conclusion was that thyroxin appears to have two separate and distinct functions: the effect upon the metabolic rate which is brought about by the CO-NH groups within the molecule; and the physiological changes involved in the metamorphosis of the tadpole due to the iodine contained in the molecule. Our own experiments have demonstrated that this action of iodine is not specific to thyroxin, but can be obtained in thyroidless amphibians (though the effects are not so rapid, and the amounts administered must be larger) by a large number of other iodine compounds and by administration of elemental iodine itself.

Kendall's experiment sheds considerable light on the reason for the conflicting results obtained by investigators working with iodine, iodized proteins and amino acids on mammals, and the students of amphibian metamorphosis. In mammals the criterion employed for testing the phsiologic action of iodine and thyroid upon the organism is the effect upon metabolism as indicated by changes in the nitrogen excretion, CO_2 elimination and oxygen consumption; in amphibians the criterion has been the rate and degree of the degenerative and regenerative processes incident to metamorphosis. However, it is becoming clear that the two types of physiologic response are not in the same class and hence not to be compared because they owe their origin to different causes. The unique effects of thyroid or thyroxin upon the meta-

bolic rate is due to the specific chemical structure of the thyroxin molecule particularly the CO–NH group, whereas metamorphic response of amphibians is dependent upon a peculiar property of iodine in certain types of combination, although not necessarily that characteristic of thyroid iodine, *e.g.*, iodized amino acids and proteins. Further evidence of the difference as to cause between metabolic changes in mammals and amphibian metamorphosis is furnished by the acetonitrile test where iodized substances shown to be specific in accelerating metamorphosis completely fail to simulate the thyroid function in protecting mice against the lethal effects of actonitrile.

Hunt and Seidell ('09) made the interesting observation that feeding thyroid tissue to white mice greatly increases the resistance of these animals to lethal doses of acetonitrile, and that the efficiency of the gland seems dependent upon its iodine content. They concluded that the increased resistance of the mice to the poison was due to the changed metabolism of the animals following thyroid feeding, the metabolic change preventing the acetonitrile from breaking down into its posionous product hydrocyanic acid. This assumption was based upon the fact that thyroid feeding does not raise the resistance of mice to lethal doses of hydrocyanic acid itself.

Koch ('13) and Miura ('22) found that iodized amino acids such as diiodotyrosine iodotryptophan and tetra-iodohistidine when administered to mice fails to increase their resistance to acetonitrile.

Strouse and Voegtlin ('09–'10) failed to observe any thyroid-like effect on the nitrogen metabolism or on the blood pressure of normal dogs, nor was there any favorable effect on the condition of myxedematous and cretinous mammals following administration of iodized amino acid. Other investigators have tried in vain to obtain thyroid effects on mammals (metabolic changes) by the use of various iodized substances; tri-iodo-imidazol and, iodophenylalanie have also proven ineffective.

If, however, we bear in mind the results of Kendall's experiment, it becomes clear why iodized amino acids, *e.g.*, iodotyrosine, give negative results when administered to myxydematous and cretinous mammals, and positive results when fed to thyroidless amphibian larvæ. In the latter group the metamorphic response

is due to the iodine in the molecule, in the former group the metabolic response depends upon something else, *i.e.*, the CO–NH group within the thyroxin molecule. This brings us to the consideration of another point, *i.e.*, the possibility of substituting other halogens for iodine and obtaining the same effects upon metamorphosis.

Kendall ('18) as a result of his investigations of the unique effects of thyroxin upon the metabolic rate of mammals, was led to conclude that insofar as the physiologic effect upon mammals is concerned, possibly other halogens could be substituted for the iodine of the thyroxin molecule without greatly changing the physiological properties of the thyroxin. His conclusion follows: "In regard to the relation of iodine to the activity of thyroxin, the presence of iodine in the compound must exert some influence, and it seems not improbable that the presence of iodine renders the active groups more reactive. In the absence of iodine it would take a greater working pressure to bring about its reaction. The substitution of iodine by hydrogen or chlorine or bromine would undoubtedly be followed by an alteration in the degree of reactivity of the substance but its gross chemical nature and properites would not be altered thereby."

Whether or not other halogens can or cannot be substituted for the iodine of the thyroxin molecule and this substance still retain its physiological activity in mammals is an open question, at any rate there are no experimental data tending to answer the question one way or the other except possibly the work of Ostwald and von Cyon who observed that thyreoglobulin containing no iodine was physiologically inert, whereas this substance gives all the physiological effects of thyroid tissue when iodine is present. However, this may be, it is clear that insofar as amphibian metamorphosis is concerned other halogens such as bromine can not be substituted for iodine. The experiments upon thyroidless axolotls and anuran larvæ where 3-5 dibromtyrosine was employed, demonstrates the futility of endeavoring to substitute bromine for iodine with hope of affecting metamorphosis. It is merely quibbling to say that bromine would probably be just as effective as iodine providing the organism possessed a mechanism for utilizing this halogen in the way the thyroid utilizes iodine to elaborate its hormone, because in our experiments the animals

fed iodotyrosine and iodoserumglobulin had no vestige of thyroid tissue present, consequently had no mechanism for iodine utilization.

No one has ever shown that in the absence of thyroid glands, other tissues of the organisms have the power of functioning vicariously for the thyroid, and synthesizing its active hormone. If this possibility were true then why is it that mammals with atrophied or degenerate thyroids are quite unable to utilize iodine or iodized proteins and amino-acids. If other tissues of vertebrates besides the thyroid glands possess the power to manufacture the thyroid hormone it is strange that this power should be present in amphibia yet lacking in mammals. The truth of the matter is that amphibian metamorphosis depends upon a peculiar property inherent in the iodine atom when combined in certain ways.

Some investigators have claimed that the pituitary can function vicariously for the thyroid when the latter is absent, hence it might be said that in thyroidless amphibians the pituitary gland may synthesize the iodine into the chemical complex characteristic of the thyroid hormone. Aside from the total lack of evidence that the pituitary can function vicariously for the thyroid in thyroidless forms, the experiments of Allen ('19) are of interest in this connection. Allen extirpated both the thyroid and the pituitary gland of frog embryos and later fed the tadpoles with starch iodide. The animals underwent a precocious and nearly complete metamorphosis before death ensued, clearly demonstrating that iodine is as effective in inducing transformation in tadpoles lacking both thyroid and pituitary as in larvæ with only the thyroid missing.

Summary of Conclusions

1. Thyroidectomized, partially thyroidectomized, and normal axolotls are readily metamorphosed by intraperitoneal injections of iodotyrosine and iodoserumglobulin and iodocasein.

2. Thyroidectomized, partially thyroidectomized and normal axolotls do not metamorphose when injected with large quantities of pure tyrosine, 3-5 dibromtyrosine (two atoms of bromine in the molecule) and non-iodized serumglobulin.

3. Larval *Spelerpes bilineatus* readily metamorphoses in strong solutions of 3-5 diiodotyrosine, but do not transform in equivalent concentrations of tyrosine and 3-5 dibromtyrosine even when kept in such solutions over comparatively long periods.

4. *Rana clamitans* tadpoles with from six to eight months of larval life remaining (*i.e.*, passing the winter as tadpoles) were metamorphosed within twenty days by rearing the animals in strong concentrations of 3-5 diiodotyrosine and feeding them with this substance. Control larvæ of similar age and developmental stage reared in equivalent solutions of 3-5 dibromtyrosine and fed quantities of the compound failed to transform.

5. The experiments are clean cut and admit of but one interpretation; it is the iodine in the amino acid and protein molecule that is responsible for amphibian metamorphosis. Why the linking of iodine to the third and fifth carbon atoms of the benzene ring of the tyrosine molecule should so greatly increase the reactive powers of the iodine is unknown.

6. Iodine other than thyroid iodine is effective in inducing the metamorphosis of thyroidless urodele and anuran larvæ. There is no evidence that any other tissue of the vertebrate organism has the power to function vicariously for the thyroid in the latter's absence.

7. Bromine has little or no influence upon amphibian metamorphosis and can not be substituted for iodine. The substitution of two bromine atoms for two hydrogen atoms (the third and fifth) of the tyrosine molecule fails to change this substance into a metamorphosis-inducing agent.

8. The physiologic responses (metabolic changes) of mammals to thyroid administration are due to the CO–NH group within the thyroxin molecule; amphibian metamorphosis to the iodine in the molecule. Consequently thyroidless animals of these two vertebrate groups are hardly comparable in regard to their response to iodine. Only thyroid iodine containing the CO–NH group is effective in thyroidless mammals whereas iodine other than thyroid iodine will metamorphose thyroidless amphibian larvæ.

9. Data are presented showing why experiments on amphibian larvæ devised to test out the effect upon metamorphosis by feeding or injecting various substances are unsound unless per-

formed upon thyroidless animals or at any rate controlled by identical experiments upon thyroidectomized individuals.

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SOME NOTES ON THE FERTILIZATION REACTION IN ECHINODERM EGGS.

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The following observations make no claim to novelty but are perhaps worthy of record as additional evidence on certain matters. They are concerned solely with the fertilization reaction, that is to say, with the activation of the egg, and not with cleavage. Activation and cleavage must, I think, be regarded as distinct processes.

The observations were made in May, 1922, at the Hopkins Marine Station, Pacific Grove, California. I am indebted to the director, Dr. W. K. Fisher, for the privilege of working at the station and for his unfailing promptness in supplying me with everything necessary for my work.

1. Materials and Methods.—The eggs used were those of the Pacific coast sea-urchins, *Strongylocentrotus franciscanus* and *purpuratus*, and of the starfish, *Patiria miniata*. The latter is the *Asterina* of Loeb. The eggs of the urchins were obtained by removing the oral portion of the test, leaving the ovaries in the aboral portion. All other viscera and tissues were then removed from the latter and it was rinsed several times with sea-water. Upon standing for a short time, those portions of the ovaries which are ripe break down, releasing the eggs, which may then be removed with a pipette. The urchins were evidently past their prime at the time during which I worked with them as only small portions of the ovaries contained mature eggs. These seemed, however, to be entirely normal in most cases, and gave a high percentage of fertilization membranes and cleavage.

The eggs of *Patiria* employed were normally shed eggs. It was found that when these starfish are spread out on a table, a considerable number of them (presumably those that happen to be ripe) will begin to shed eggs and sperm and continue this for three or four hours. It may be noted in passing that the normally shed eggs of *Patiria* are fully mature, are immediately

fertilizable as they exude from the genital pores, and yield 100 per cent. fertilization membranes, cleavage, and larvæ. This agrees with observations on other starfish. As is well known, and this is also the case in *Patiria*, starfish eggs obtained by shaking or mincing the ovaries are not mature, but must stand in sea-water for some time before they attain a fertilizable condition.

In the following account a number of statements are made concerning viscosity differences. These have been determined in the following manner. The eggs are placed on a slide in a drop of sea-water without cover. Low power was used (Leitz objective 2, ocular 3). Any egg to be investigated was rapidly pushed with a needle to the periphery of the drop and then into a small evagination of the periphery where it is held by surface tension. It was then punctured or cut with a needle and the rate and readiness with which the egg cytoplasm flows out under the pressure of surface tension as well as the duration of retention of cuts and gashes furnish a relative measure of the viscosity of the cytoplasm. The needle used was a fine steel needle thrust into a wooden handle and operated by hand. The method is somewhat crude but has the advantages that it is rapid and almost entirely objective.

2. *Viscosity of the Unfertilized Mature Egg.*—The unfertilized egg in all three species consists of a slightly viscous cytoplasm inclosed in a definite membrane of considerably greater consistency than the cytoplasm. This membrane, which may be designated the vitelline membrane (it has also been named plasma membrane and egg membrane) is probably a colloidal gel. It is more delicate in the sea-urchin than in the starfish egg.

These facts have been determined as follows. When the sea-urchin egg (either species) is held by surface tension and punctured with a needle, the cytoplasm rushes out with almost explosive force and the whole egg very rapidly disintegrates. In this disintegration the vitelline membrane is also involved so that I at first thought such a membrane was absent. However, if the eggs are rapidly pushed back into the drop when they are partially disintegrated, egg fragments of various sizes are obtained. On the surface of such fragments wrinkles are always observable. It is evident that such wrinkles must be located in a surface membrane of greater consistency than the cytoplasm,

in fact, of solid consistency, as fluids do not exhibit permanent wrinkles. These wrinkles on egg fragments are illustrated in Figs. 10 and 13. In only one case did the entire membrane persist after disintegration of the cytoplasm; this case is illustrated in Fig. 16. The membrane is collapsed and wrinkled.

In the *Patiria* egg, when punctured as just described, the cytoplasm flows out invariably leaving the membrane behind. This is illustrated in Figs. 19 to 25. From the fact that the membrane always persists in the starfish egg after disruption of the cytoplasm and rarely so persists in the urchin egg, I have drawn the conclusion that the vitelline membrane of the former egg is firmer, tougher, and probably thicker than in the latter egg. This difference is further evidenced by the fact that the empty membrane in *Patiria* retains its former shape better than in the urchins as may be seen by comparing Figs. 16 and 19.

The consistency of unfertilized eggs has been previously described by a number of investigators. The first detailed description of the viscosity conditions in unfertilized eggs seems to have been that of Herbst ('93). Herbst noted that if pressure is applied to unfertilized sea-urchin eggs, the contents flow out and a fine membrane is sometimes left behind to which bits of protoplasm still cling. He therefore concluded that the surface layer of the egg possesses a greater consistency than the remainder of the egg but is not definitely separated from the latter. Lillie ('06) notes that the *Chætopterus* egg is semifluid and surrounded by a delicate membrane. Heilbrunn ('15) states that the cytoplasm of the unfertilized *Arbacia* egg is "typically fluid" and inclosed in a membrane described as being "a protein gel" and possessing "a certain degree of rigidity." Chambers ('17*a*) finds that the protoplasm of the unfertilized eggs of *Arbacia*, *Asterias*, *Echinarachnius*, *Cerebratulus*, and *Fucus* is a "hyaline fluid" of "very slight consistency" while the surface layer is "very dense in consistency as compared with the cell interior into which it merges insensibly." Chambers has also noted the greater delicacy of the membrane of the urchin egg than of the starfish egg ('21*b*). Heilbrunn ('20*b*) describes the *Cumingia* egg as "a mass of fluid protoplasm surrounded by a rigid membrane." Seifriz ('18, '20) finds that ripe *Fucus* eggs are decidedly viscous with a wall consisting of a very rigid hyaline gel; he also describes

the cytoplasm of *Tripneustes* and *Echinarachnius* eggs as of about the consistency of glycerine. The cytoplasm of the eggs which I have investigated seems to me to correspond to 4 or 5 in Seifriz's scale ('20, p. 364). The existence of a membrane around the unfertilized egg has also been asserted by many other investigators apart from considerations of viscosity differences.

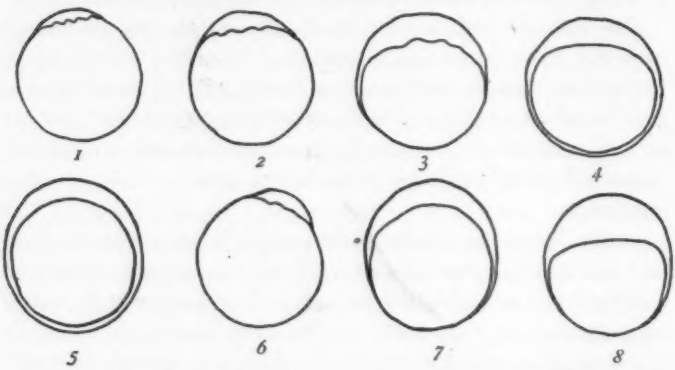
The vitelline membrane is probably not sharply delineated from the less viscous egg cytoplasm. According to Chambers ('17*a*) the external jellied surface of the egg cell "gradually passes into the sol of the interior." This gradation must be very abrupt as I did not notice it in my experiments although I think the superficial cytoplasm does undoubtedly adhere to the vitelline membrane.

3. The Morphology of the Fertilization Process.—The fertilization reaction has been observed on eggs mounted in plenty of water without a cover glass; on eggs in a depression slide with a cover glass; and on eggs in a hanging drop on the under surface of a cover glass placed over a depression. The reaction has been studied with low, medium, and high powers. The phenomena observed were the same by any method of mounting the eggs and cannot possibly be ascribed to compression, for the eggs were never under compression.

The fertilization reaction begins to be visible 45 to 60 seconds after mixing the eggs with dilute sperm suspension. It is first indicated by a roughening or crenation of the egg at one place on the surface and this roughening spreads very rapidly in all directions from the initial place over the entire surface of the egg. Following closely upon this change the vitelline membrane begins to elevate. This elevation starts at the same place as the roughening and like the latter sweeps in all directions over the egg. The roughening and the elevation of the membrane occur at such a close interval as to be almost simultaneous but it is certain that the former precedes. There is also to be noted a flattening of the egg at the region of initial membrane elevation. This flattening is commonly very marked at the site of initial membrane elevation and spreads from this for a short distance but never extends more than halfway over the egg. The fertilization changes in *S. franciscanus* are illustrated in Figs. 1 to 5.

The time required for these changes to pass over the egg is

about 15 to 30 seconds in the best eggs. In subnormal eggs the time is much longer and the reaction may not be complete, the eggs remaining permanently with membranes partially elevated as shown in Figs. 6 to 8. Such cases serve, I think, to indicate further the progressive character of the fertilization reaction.



All figures are redrawn from free-hand sketches. In the unfertilized egg the vitelline membrane is not indicated separately from the egg surface as doing so would exaggerate the real appearance.

FIGS. 1 TO 5. Five stages in the normal fertilization reaction in *Strongylocentrotus franciscanus*. Note progress of the fertilization reaction from the initial place. In 5 the reaction is not yet quite complete as the egg is still slightly excentric within the membrane.

FIGS. 6 TO 8. Permanent stages of partial fertilization from a subnormal lot of eggs of *S. franciscanus*.

In eggs favorably placed for such observation it has been determined that the point on the egg from which the fertilization changes take their origin is the place to which the successful sperm is attached. This has also been ascertained by so many previous observers, to whom reference will be made shortly, that it seems superfluous to dwell upon the fact. The fertilization reaction begins at the point of contact of sperm and egg and from this point is transmitted in all directions over the surface of the egg.

The fertilization reaction is essentially the same in all three species studied. The chief difference between the urchin egg and the starfish egg is that in the former the membrane where it first separates from the egg elevates at once in this region to its

fullest extent whereas in the starfish egg the membrane separates from the entire surface of the egg before it elevates to any considerable extent (Fig. 9). Consequently in the urchins the egg at first lies very asymmetrically placed within the membrane (Figs. 4 and 5). This asymmetry is further emphasized by the flattening of the egg at the region of initial elevation. Later symmetry is restored in normal eggs by the widening of the perivitelline space at other regions and by the resumption of spherical form by the egg. In subnormal eggs the asymmetry is likely to persist. In the *Patiria* egg, the membrane separates from the whole surface of the egg with the formation of only a very narrow perivitelline space; later this widens simultaneously around the egg. The flattening at the region of initial membrane elevation is slight in the starfish egg but generally perceptible.

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FIG. 9. Stage in the fertilization reaction of *Patiria miniata*. The vitelline membrane separates only slightly from the egg at first. Note also the crenation of the egg surface.

The separation of the vitelline membrane does not proceed with entire smoothness from the point of initiation but the membrane tends to adhere to the egg surface at some points slightly longer than at others. An exaggeration of this tendency results in the vesicle formation described and figured by Loeb ('13), observable according to him through retarding the normal reaction by lowering the temperature. Vesicle formation also occurs at ordinary temperatures in subnormal eggs and in such eggs the vesicles may persist, not flowing together to form a continuous perivitelline space. Pronounced vesicle formation seems to be due to a subnormal response of the egg to fertilization.

An attraction cone at the site of sperm entry such as has been reported by others was not noticed but was not particularly sought for. I was also unable to observe the passage of materials from the cortex into the perivitelline space, as described by Just ('19) for *Echinarachnius*, although I searched carefully for such a process.[1]

[1] In making this statement I do not intend to imply the slightest question of the validity of Just's observations. I believe from other lines of evidence that material does pass from the egg into the perivitelline space but the process is invisible in the eggs with which I have worked.

The progressive character of the fertilization reaction has been previously described by a number of investigators. It was first noticed about simultaneously by Fol ('77, '79) and Calberla ('78). They observed that the detachment and elevation of the fertilization membrane are initiated at the point of attachment of the successful sperm and proceed from there in all directions around the egg. Fol [1] described this process for *Asterias glacialis*, *Toxopneustes lividus*, and *Sphærechinus brevispinosus*. Calberla's investigations concern the *Petromyzon* egg. This egg possesses a micropyle; no change occurs until the sperm has passed the length of the micropyle and touched the surface of the egg cytoplasm inside the membrane. When this happens the egg withdraws a little from the vitelline membrane at the micropyle and flattens slightly; the separation of the membrane from the egg followed by its elevation then proceeds from the micropyle over the egg. Théel ('92) describes the elevation of the membrane in the sea-urchin *Echinocyamus* in the following words: "At the place where the first sperm has penetrated the mucilaginous investment, a very thin plasmatic membrane rises and separates from the egg, beginning at the place of contact and extending eventually around the yolk." Herbst ('93) agrees with Fol's description of the fertilization reaction in the sea-urchin egg. Ries ('09*a*) also observed the progressive character of membrane elevation in the sea-urchin egg and presents photographs of the process, taken with a motion picture machine. Elder's drawing of the elevation of the fertilization membrane in *Strongylocentrotus purpuratus* shows that this process is a progressive change initiated at one place but Elder does not mention this in the text ('12). Okkelberg ('14) has described the elevation of the membrane in the egg of the brook lamprey *Entosphenus wilderi*. In this egg fertilization occurs at the animal pole and the membrane here separates from the egg. A wave of contraction then passes over the egg towards the vegetative pole, separating the membrane from the egg. Just ('19) has given a careful description of the fertilization reaction in *Echinarachnius parma*. The reaction

[1] There seems to be a prevailing impression that Fol worked with compressed eggs. This is not, however, the case as Fol was at particular pains to state that the eggs were not in the least compressed ("sans les comprimer le moins du monde," '79, p. 176).

is similar to that in other echinoderm eggs. "The cortex reacts to penetration by pushing out a blister at the site of sperm entry." "From the point of sperm entry a definite gradient of membrane elevation is established, the last point of membrane elevation being at the pole opposite that of successful sperm entry."[1]

It therefore appears that in many eggs—echinoderms and lampreys—a change in the surface of the egg is initiated by contact of sperm and egg and that this change, which includes membrane separation and elevation, progresses from the point of contact in all directions to the opposite pole of the egg. It is probable that more careful observation would reveal the progressive character of the fertilization reaction in other eggs.[2] The wave-like progression of the reaction irresistibly suggests that electric phenomena are involved; recently Gray ('22) has made the same suggestion.

The roughening of the egg at fertilization has also been noted by a few observers. Schücking ('03) and Loeb ('13) record it for echinoderm eggs. The "peristaltic wave" which according to Okkelberg ('14) passes over the lamprey egg in normal fertilization or artificial activation is, I think, of the same character. Mr. Leigh Hoadley informs me that a roughening also occurs in the *Arbacia* egg on fertilization. The cause of the roughening is discussed later.

Flattening of the egg at the site of sperm entry has also not escaped observation and is regarded by some as a contraction. Hertwig ('78) probably has reference to this flattening when he states that in the starfish egg on fertilization "zieht sich der Dotter von der Eihaut zurück." Some of Fol's figures ('79) show this flattening and it is also recorded for *Toxopneustes* by Selenka ('78) and for *Petromyzon* by Calberla ('78). Schücking ('03) speaks of a contraction of the egg away from the vitelline membrane and probably has reference to the same phenomenon. The photographs of Ries ('09*a*) of fertilization in *Strongylocentrotus* plainly show the flattening at the site of initial membrane elevation and this is also mentioned by him in the text. Elder's

[1] Professor F. R. Lillie informs me that while at Pacific Grove in the winter of 1920 both he and his assistant, Mr. J. Nelson Gowanlock, observed the progressive character of the fertilization reaction in *Strongylocentrotus*.

[2] But not in teleost eggs, according to Reighard '93.

drawing ('12, Fig. 6) of fertilization in *S. purpuratus* is similar. Gray ('16) speaks of the compression of the egg at fertilization by the contents of the perivitelline space.

Watching the fertilization process one certainly gains the impression that the flattening of the egg is due to a pressure exerted on the egg by the contents of the perivitelline space. It appears that the filling of this space does not occur *pari passu* with the elevation of the membrane but is due to some other process. This suggests that the accumulation of materials in the perivitelline space is the direct cause of the elevation of the membrane and such an idea has been advanced by many investigators.[1] Further evidence on this matter is desirable.

4. The Identity of the Vitelline Membrane with the Fertilization Membrane.—This matter has been the subject of some dispute in the history of the fertilization problem. Harvey ('10, '14), McClendon ('11), Elder ('12), Loeb ('13), and recently Gray ('22) have expressed the view that the fertilization membrane is formed by the precipitation or coagulation of materials emanating from the egg on contact with the jelly or the sea-water. That the jelly is not concerned in the appearance of the fertilization membrane has been shown by Harvey ('14) and Lillie ('14, p. 553).

On the other hand the identity of the fertilization membrane with the preëxisting vitelline membrane of the unfertilized egg has been maintained by many investigators: Fol ('77, '79) for *Asterias*, *Sphærechinus*, and *Toxopneustes*, Hertwig ('78) for *Asterias*, Calberla ('78) for *Petromyzon*, Théel ('92) for *Echinocyamus*, Reighard ('93) for teleost eggs, Herbst ('93, '04) for *Sphærechinus*, *Echinus*, and *Stronglyocentrotus*, Delage ('01) for *Asterias*, Schücking ('03) for *Asterias* and *Strongylocentrotus*, Ries ('09*a*) for *Strongylocentrotus*, Allyn ('12) for *Chætopterus*, Glaser ('13) for *Arbacia* and *Asterias*, Heilbrunn ('13, '15, '20*b*) for *Arbacia* and *Cumingia*, Okkelberg ('14) for *Entosphenus*, and Chambers ('21*a*, '21*b*) for *Arbacia*, *Asterias*, and *Echinarachnius*. Most of these authors rest their view on direct observation of

[1] *E.g.*, Herbst '93, '04, Schücking '03, Ries '08, '09*a*, Loeb '08. It seems clear that the contents of the perivitelline space consist chiefly of water which enters from the outside through the vitelline membrane. This was proved for the lamprey egg by Calberla ('78) by coloring the water and is certain for teleost eggs (Reighard '93). The perivitelline space also appears to contain some colloidal material probably of protein nature derived from the egg.

the elevation of the vitelline membrane as the fertilization membrane. Such evidence is not entirely satisfactory for the echinoderm egg as the vitelline membrane is not clearly visible on such eggs. Chambers' evidence appears to be conclusive. He has shown that if the vitelline membrane be removed from unfertilized eggs (*Arbacia*, *Asterias*, *Echinarachnius*), such eggs do not elevate membranes on fertilization. Further by various methods the vitelline membrane can be made more obvious on the unfertilized eggs at certain points and the continuity of these easily visible portions of the membrane with the fertilization membrane after insemination is easily observable.

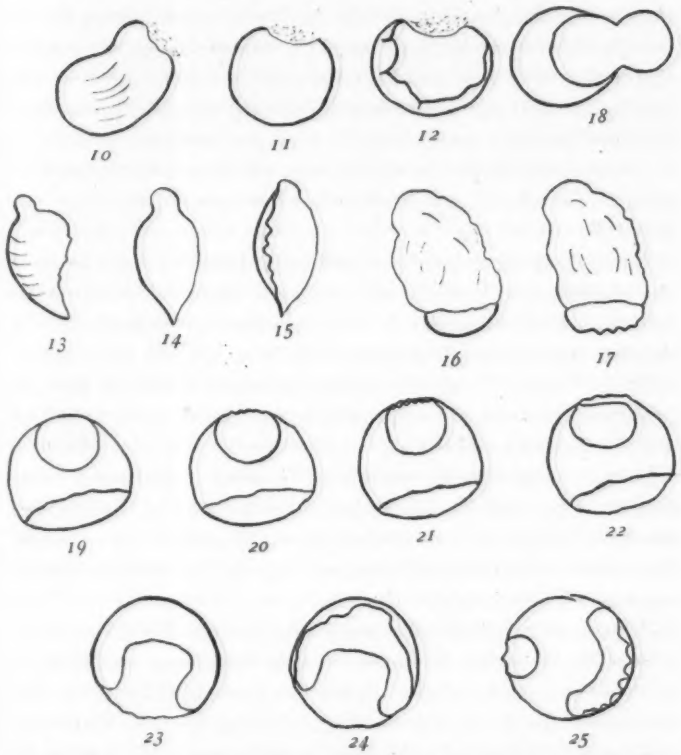
Further conclusive evidence of the identity of the vitelline and fertilization membrane is afforded by the study of the fertilization reaction in egg fragments. That portions of eggs may elevate fertilization membranes has been noted by Ziegler ('98), Moore ('12) and Glaser ('13). The fragments in these experiments were obtained by shaking the eggs and hence it is not known from which portions of the eggs they originate. It is certainly not justifiable to conclude from such experiments that any portion of the egg when isolated can reform a membrane capable of elevation since: (*a*) the cytoplasm being relatively fluid, much of it will be lost on rupture of the eggs and the cortical portion which adheres to the membrane will be most likely to persist; and (*b*) since it has been proved by Chambers ('21*a*, '21*b*) and Just ('23) that the interior of the egg is not fertilizable but only the cortex possesses this property, it follows that any fragments which fertilize must necessarily have retained a portion of the egg cortex and hence of the original vitelline membrane. It seems likely that most of the egg fragments obtained by shaking represent the whole cortex, the fluid interior having escaped.

I have studied the fertilization reaction in fragments of the eggs of all three species used. These fragments have been obtained by the method already described. An egg is held by surface tension and then punctured. The cytoplasm flows out and the egg begins to disintegrate. At various stages in this process the egg is rapidly pushed back into the interior of the drop. The outflow at once ceases when the pressure of surface tension is removed and portions of eggs of all sizes and shapes are obtainable. Such fragments of course always retain a portion of the

original cortex and vitelline membrane and it is definitely known to the observer which is the original and which the new surface of the fragment. In *Patiria* the whole vitelline membrane clings to the fragment. The fragments show little tendency to assume the spherical form.

Upon the addition of sperm all such fragments give evidence of the fertilization reaction. The first change noted is an alteration of tension in the fragment. This is evidenced by the disappearance of the wrinkles from the vitelline membrane of fragments of urchin eggs and by a change of shape. The fragments tend to assume a more spherical form. This tendency towards sphericity is very marked in the larger fragments as in Figs. 10 and 11. From these observations the conclusion can be drawn that the change of tension is confined to the original cortex, since when the amount of cortex remaining is small there is much less change in the shape of the fragment than when it is large. The next change that is noted is the roughening of the surface. This occurs *only on that surface of the fragment which was the original surface of the egg*. In the smaller fragments the visible fertilization change stops at this point. The membrane follows the crenations of the surface as shown in Figs. 17 and 20; consequently the crenations cannot be due to the formation of small vesicles as supposed by Loeb ('13). In the larger fragments the roughening of the surface is followed by the elevation of the fertilization membrane. The membrane elevates *only on that surface of the fragment which was the original surface of the egg* and which therefore bears a portion of the original vitelline membrane. In the *Patiria* egg where the whole vitelline membrane remains attached to the fragments, the membrane elevated on the fragment is perfectly continuous with the empty portions of the vitelline membrane as shown in Figs. 19 to 25. The elevation of the membrane on egg fragments is illustrated in Figs. 10 to 25.

The fertilization reaction in eggs with extra-ovates is to the same effect. Glaser ('13) records that if the egg of *Arbacia* is ruptured so that part of the contents flows out, a fertilization membrane appears on one sphere but not on the other. According to my observations on the same egg, it is the cortical sphere which elevates a membrane. Chambers ('21*a*, '21*b*) also finds that extra-ovates in the starfish are not fertilizable and do not



FIGS. 10 TO 25. Fertilization of egg fragments. The original surface is indicated by a heavier line. Figs. 10 to 18, fragments of *S. franciscanus*. Fig. 10, large egg fragment showing wrinkles in the vitelline membrane; Fig. 11, same after insemination; the fragment is rounded and the wrinkles have disappeared; Fig. 12, same later, with membrane elevated on the original surface. Fig. 13, smaller fragment; Fig. 14, same after insemination, showing change of form and disappearance of wrinkles; Fig. 15, same, later, with fertilization membrane elevated on original surface only. Fig. 16, small fragment with entire membrane attached; Fig. 17, same after fertilization; only the characteristic crenations appear; the membrane does not elevate on such small fragments. Fig. 18, extra-ovate of *S. franciscanus* after fertilization; cortical sphere to left, endoplasmic sphere to right; only the former elevates a membrane.

FIGS. 19 TO 25. Fertilization of egg fragments of *Patiria miniata*. Fig. 19, small fragment with entire vitelline membrane; Fig. 20, same after fertilization, showing the characteristic crenations; Figs. 21 and 22, same, later, showing separation of the fertilization membrane; it is continuous with the vitelline membrane. Fig. 23, large fragment of *Patiria;* Fig. 24, same after fertilization. Fig. 25, large and small fragment left within the same vitelline membrane, shown after fertilization; the small fragment exhibits only the characteristic crenations; the large one has elevated a fertilization membrane.

elevate fertilization membranes but that these processes are confined to the cortical portion of the ruptured egg. He further states that if the extra-ovate remains in continuity with the cortical portion for some time it becomes fertilizable but still does not elevate a membrane. I have produced extra-ovates in *S. franciscanus* with the needle; upon fertilization the cortical portion elevates a membrane while the extra-ovate does not (Fig. 18).

From all of these observations it may be concluded: (*a*) that the vitelline membrane is identical with and is elevated as the fertilization membrane; (*b*) that the vitelline membrane is a definite morphological structure which cannot be reformed or replaced;[1] and (*c*) that the fertilization reaction, which includes other visible changes besides membrane elevation, is exhibited only by the cortex of the egg.[2]

Since the fertilization membrane is thus a preformed, pre-existent structure, its separation from the egg at fertilization should be spoken of as membrane elevation and not as membrane formation. The advisability of this terminology was previously emphasized by Heilbrunn ('15).

When the vitelline membrane elevates as the fertilization membrane, however, it unquestionably undergoes certain physical and chemical changes. In the first place it would seem that the membrane must soften since in elevating it is distended considerably beyond its former circumference. A number of observers have attested that there is no decrease in size of the echinoderm egg at fertilization (Fol '79, Théel '92, Herbst '93, Schücking '03, Ries '08, Loeb '08, McClendon '10, Chevroton and Vlès '11, Gray '16, Chambers '21*b*). I was also unable to find any change in size of the eggs of *S. franciscanus* on fertilization.

[1] Any portion of the egg cytoplasm does of course when exposed to sea-water form a protective surface layer which has no doubt physiological properties similar to those of the vitelline membrane but which is not elevatable by sperm. Several observers (Herbst, '03, Tennent and Hogue, '06, Harvey, '10, Heilbrunn, '20*b*) have recorded that a second membrane can be elevated after the elevation of the usual fertilization membrane by treatment with various agents (but not with sperm). This second membrane is in reality the hyaline layer, as also stated by Harvey ('14) and Heilbrunn ('20*b*).

[2] The importance of the cortex finds explanation on the basis of Lillie's fertilizin theory (cf. Lillie, '19, also Chambers, '21*b*). Just ('23) also shows that the fertilization reaction is confined to the cortex.

Consequently the vitelline membrane is distended at fertilization, and such distension indicates a softening or degelation of the membrane. According to R. S. Lillie ('15) the temperature coefficient of heat activation in the starfish egg indicates that a degelation process is involved. Just's ('22) recent experiments show that the membrane [1] becomes very much less resistant at the places where it is separating from the egg for such places burst when the egg is exposed to diluted sea-water during elevation. Thus the decreased resistance or softening of the vitelline membrane like other fertilization changes is initiated at the point of contact of sperm and egg and is transmitted from this point over the egg. Within a few minutes after elevation the membrane appears to toughen again. This hardening or toughening of the membrane after elevation has been spoken of by several observers. Thus Herbst ('93), Goldfarb ('13), C. R. Moore ('16), F. R. Lillie ('21) have recorded that the fertilization membrane of urchin eggs is much more easily removed by shaking within a few minutes after fertilization than later. Chambers ('21*b*) also finds that the fertilization membranes of *Asterias*, *Arbacia*, and *Echinarachnius* begin to toughen very soon after they are elevated. The extensive hardening of the fertilization membrane in nematode eggs is well known. That the membrane also undergoes chemical alteration on elevation may be inferred from an experiment of Harvey's ('10). He found that the vitelline membrane is soluble in concentrated sulphuric acid while the fertilization membrane is not.

5. Viscosity Changes at Fertilization.—It was pointed out in the first part of this paper that the cytoplasm of the unfertilized egg is a slightly viscous fluid. It can be determined by the same procedure as described there that at fertilization there is a sudden and marked increase in viscosity. In the eggs of *Strongylocentrotus* (both species) this increase in viscosity occurs just before the membrane begins to elevate; this is probably also true of the *Patiria* egg although it was not determined with certainty. It is present in this egg at least as soon as the membrane lifts at the site of sperm entry. The viscosity increase is

[1] Just speaks only of the softening of the cortex but probably includes the membrane in this term. It seems evident that the membrane must also be involved in the softening otherwise it would not yield at the places in question.

well marked and occurs very suddenly, at the moment before membrane elevation is initiated. At this moment, the cytoplasm flows less readily than before and cuts and gashes made with the needle close up more slowly. The viscosity increases during the elevation of the membrane but appears to reach a maximum in a very short time.

We may therefore speak of an increased viscosity or gelation of the egg cytoplasm as one of the changes included in the fertilization reaction. I believe this gelation to be responsible for the roughening of the egg surface at fertilization. As Rhumbler ('05) has recognized, a rough and crenated surface is causally related to a solidified condition of protoplasm. As the roughening of the egg begins at the point of contact of egg and sperm and spreads from there over the egg, it follows that the gelation process must likewise originate at the site of sperm entry and progress from this point in all directions. It seems probable that the gelation process is confined to the cortex of the egg since in egg fragments only the original surface roughens at fertilization.

The interpretation of the rounding up of egg fragments remains to be considered.[1] I at first thought this to be another indication of increased viscosity, of the increased tension accompanying the change from a more fluid to a more viscous state. But obviously gelation cannot cause both a rounding up and a roughening. Although the surface tension of gels is higher than that of sols, still surface tension is not great enough to induce sphericity in fragments of gels. It therefore seems necessary to conclude that the rounding of egg fragments indicates a decreased viscosity or increased fluidity. As my observations show that this process precedes the roughening by a quite perceptible time interval, it seems that the cortex of the egg at fertilization first becomes more fluid and then undergoes gelation. Just ('22) emphasizes a liquefaction of the cortex as part of the fertilization reaction.

The gelation of the egg at fertilization serves at least two purposes: (*a*) the vitelline membrane is split from the egg cyto-

[1] Harvey ('10) has also emphasized the rounding up of eggs at fertilization and attributes it to an increase in tension. However, the same tension will induce sphericity if the cytoplasm becomes more fluid. My observations on egg fragments indicate that this change in tension is in the cortex, not in the membrane.

plasm and acquires a definite internal boundary which it had hitherto lacked;[1] and (*b*) a new surface is formed on the egg cytoplasm, the so-called hyaline layer, which prevents the egg contents from expanding with the membrane and which replaces physiologically the vitelline membrane. The separation of the vitelline membrane from the egg cytoplasm at fertilization thus appears to me to be a process quite independent of the subsequent elevation of the membrane. The hyaline layer which replaces the vitelline membrane requires some time for its complete development and is according to Chambers ('21*b*) "firm and gelatinous."

An increased viscosity accompanying the fertilization reaction has not been hitherto recorded. Several observers have, however, noted such an increase following after fertilization, recently Heilbrunn ('15, '20*a*, '21), Chambers ('17*b*, '19), and Seifriz ('20). According to these investigators the increased viscosity is associated with some phase of the mitotic figure. According to Chambers ('17*b*) the gelation after fertilization is at first limited to the small sperm-aster and later spreads throughout the egg. While fully accepting the conclusion of these authors that asters and spindles are gelation figures I do not think that the initial gelation which constitutes part of the fertilization reaction is due to the sperm aster. The latter is at first localized around the sperm head while the gelation which I am considering appears to be general throughout the whole cortical region of the egg.

Since the viscosity changes at fertilization precede the elevation of the membrane it may be emphasized that the latter is a secondary rather than a primary phenomenon in the fertilization reaction. It seems certain that changes have taken place in the egg before the membrane elevates. This has recently also been emphasized by Just ('19): "In the *Echinarachnius* egg, normal development has already been initiated by the sperm when the membrane begins to form." It appears that the elevation of the membrane is not due directly to sperm penetration but is the result of changes in the egg.

6. Artificial Membrane Elevation and Cytolysis.—It is well

[1] Fol ('79) in particular emphasizes that the vitelline membrane (couche enveloppante) lacks a definite internal boundary and that it acquires one at fertilization.

known that a number of chemical substances as well as other agents will induce a fertilization membrane in echinoderm eggs. This matter has been discussed from the point of view of membrane formation by Traube ('09), Loeb ('13), Gray ('22) and others and from the point of view of membrane elevation by Heilbrunn ('13, '15). I have made a few observations on artificial membrane elevation, using chiefly ether and diluted sea-water or distilled water but also butyric acid. In employing ether and similar substances for membrane elevation, the eggs must be rapidly returned to normal sea-water, as noted by Loeb ('13) if any are to be saved from cytolysis.

After the application of membrane-elevating substances such as ether and distilled water, three classes of eggs are noted: those with blister-like elevations, those with completely elevated membranes, and those which are cytolyzed. In a considerable number of eggs after treatment with ether and distilled water, the membrane is elevated only as local blisters. In one or two cases such blisters were observed to spread over the egg until the entire membrane was elevated but generally they persist unchanged as long as observed (unless cytolysis intervenes). They appear to be due to chance inequalities of contact with the membrane-elevating solution when the latter is first applied and indicate that the local action of such solutions is incapable of inducing complete membrane elevation. Such partially elevated membranes can be completed by sperm. The portions elevated by sperm appear to be continuous with those elevated by the agent.

After treatment with ether and distilled water there is obtained a small percentage of eggs in which the membrane is completely elevated and which cannot be distinguished visibly from eggs fertilized by sperm. Investigation with the needle shows, however, that as concerns viscosity conditions these eggs are entirely different from normally fertilized eggs. Whereas in the latter, as already noted, the cytoplasm has undergone gelation, in these eggs with artificially elevated membranes there is no trace of such increased viscosity. These latter eggs are as fluid as or more fluid than normal unfertilized eggs. This agrees with Heilbrunn's ('20*c*) statement that ether, chloroform, and similar

substances liquefy the *Arbacia* egg. It is thus evident that the response to membrane-elevating substances, so far as tested, is not equivalent to the response to sperm.

The usual butyric acid treatment yields, after return to normal sea-water, a percentage of eggs with membranes of normal appearance equal to that produced by sperm. But in such eggs, also, the normal gelation appears to be lacking.

The majority of the eggs treated with membrane-elevating substances such as ether and distilled water undergo a change designated by Loeb as cytolysis.[1] This condition is sufficiently described in Loeb's book ('13). The eggs are much expanded and transparent. It appears from Loeb's description ('13, p. 188) that he regards this cytolysis to consist in an absorption of fluid followed by a liquefaction of some of the egg contents. It can readily be shown by the needle that this conception of cytolysis is erroneous. Cytolysis is not a liquefaction of the egg contents; it is a complete and irreversible coagulation. This fact was discovered by Heilbrunn ('15) and the increased viscosity of cytolyzed eggs was also noted by Goldfarb ('18). When cytolyzed eggs are punctured with a needle, a small amount of watery fluid generally escapes (this is probably water which passes in from the outside when the membrane expands) but the egg material itself will no longer flow. It is completely solidified and can be cut into pieces with a needle.

The coagulation[2] caused by membrane-elevating solutions is entirely different from the normal gelation attendant on fertilization. The cytolytic change is an irreversible lethal change in which the egg colloids are precipitated out in a coagulated mass. The viscosity of the cytolyzed egg is very much greater than that of the normally fertilized egg at any time from fertilization to the first cleavage. The normal gelation on the other hand is a reversible physiological process in which there is no such precipitation of colloids.

The cause of cytolysis appears to be as follows. Since after

[1] My remarks refer only to the "white" cytolysis of Loeb.

[2] The term coagulation can of course be used to designate any marked increase in viscosity. It seems preferable to me, however, to confine the term to an irreversible separation out of colloids and to use the term gelation to designate reversible physiological increases in viscosity.

treatment with membrane-elevating solutions without cytolysis, the membrane elevates without an accompanying cytoplasmic gelation, it is evident that the egg is left without a resistant surface. Consequently the surface of the egg after lifting of the vitelline membrane is weak. The membrane-elevating substances tend to induce the expansion of the egg in the same way as they induce the expansion of the membrane (cf. further Heilbrunn, '15) and as the egg is not protected by a resistant surface it naturally ruptures by expansion. Such rupture causes coagulation since it has been shown by the microdissectionists (Chambers, '17*a*, Seifriz, '20) that injury leads to coagulation. The agents in question do not cause coagulation directly but act by elevating the protective vitelline membrane from the egg, leaving the cytoplasm without a sufficiently resistant surface.

From these considerations it is highly questionable whether the cytolytic action of parthenogenetic agents has any relation whatever to their parthenogenetic power or whether any conclusions can be drawn concerning the mechanism of normal activation from the cytolytic properties of such agents. A similar conclusion as to the lack of relation between cytolysis and activation has been reached by Just ('20) from other lines of evidence. It seems sufficiently evident that cytolysis is simply a death change and has no bearing on activation.

It occurred to me to determine whether artificial agents can elevate membranes on eggs in which the sperm are not able to do so. It is well known that after standing in sea-water for twenty-four hours or more urchin eggs no longer elevate membranes on fertilization although they are still capable of development. The vitelline membrane probably loses its elasticity and capacity for distension after a prolonged stay in sea-water. I found a considerable degree of parallellism between the action of sperm and of artificial agents on such eggs. It is much more difficult and in some cases impossible to induce membrane elevation by agents in eggs which do not elevate membranes on insemination. In most cases, however, the artificial agents are more or less effective in partially or completely elevating the membrane. The action of these agents is thus more powerful than that of the sperm and may possibly be of a different nature.

In some cases membrane elevation could not be induced by artificial agents in these stale eggs. In such cases the egg bursts through the membrane, forming either a number of vesicles or erupts after the manner of an extra-ovate. This further indicates that the agents used have an expansive effect upon the egg contents as well as on the membrane. When extra-ovates are formed from stale eggs by these agents the vitelline membrane separates from the egg contents. As this is not the case when extra-ovates are induced in fresh eggs, one may again conclude that the vitelline membrane loses its elasticity and distensibility on standing. The membrane appears much stiffer and firmer than in fresh eggs; in all probability it is coagulated.

7. *Conclusion.*—In conclusion I may be permitted to reiterate an old view that the activation of the egg by the sperm is of the nature of a stimulation or excitation. Among the characteristics of a stimulation are: (*a*) many different agents are capable of exciting the same effect in the protoplasm which is stimulated so that there is no specific relation between the properties of the agents and the change invoked in the protoplasm; (*b*) the changes induced in the protoplasm stimulated are altogether in excess of the energy content of the stimulus; (*c*) the changes invoked in the stimulated protoplasm depend upon the constitution of the protoplasm and not upon the nature of the stimulus; *d*) the excitation is transmitted from the point of application of the stimulus. It is evident that the activation of the egg exhibits these characteristics. Many different agents are able to induce activation. There appears to be no specific relation between the changes induced in the egg and the physical and chemical properties of the stimulating agents. To suppose that the sperm brings into the egg some substance which evokes the activation changes is I think just as far from the truth as to suppose that the various agents which induce a nerve impulse do so by injecting some substance into the nerve. I think one must agree fully with Lillie ('19, Chap. VII) that the egg is "an independently activable system" and "possesses all of the substances necessary for activation." Finally it has been shown in at least a number of cases that the fertilization reaction is initiated at the point of sperm entry and is transmitted from this place over the egg. The

transmitted changes recorded in this paper are: the roughening (gelation) of the egg and the elevation of the fertilization membrane. To these may be added those recorded by Just ('19, '22)—the loss by the egg of fertilizibility, the passage of materials from the cortex into the perivitelline space, and the softening of the vitelline membrane.

The problem of the activation of the egg becomes thus a problem of the nature of stimulation in general and cannot be solved until the more general problem has attained solution.

8. Summary.—(*a*) The eggs used were those of *Strongylocentrotus franciscanus* and *purpuratus* and *Patiria miniata*.

(*b*) Physically these eggs consist of a slightly viscous cytoplasm inclosed in a vitelline membrane of solid consistency.

(*c*) In all three species the fertilization reaction begins at the point of attachment of the successful sperm and is transmitted from this place in all directions over the egg.

(*d*) The visible manifestations of the fertilization reaction are a roughening of the surface and the elevation of the vitelline membrane. Both begin at the site of sperm entry and spread from there over the egg.

(*e*) The vitelline membrane is identical with and is elevated as the fertilization membrane.

(*f*) In fragments of eggs only that surface of the fragment which was part of the original surface of the egg shows the fertilization reaction-roughening and membrane elevation.

(*g*) The vitelline membrane cannot be replaced or reformed.

(*h*) At the moment of fertilization just preceding the elevation of the vitelline membrane an increased viscosity or gelation occurs in the egg. This splits the vitelline membrane from the egg cytoplasm and provides a new resistant surface for the latter. This gelation is the cause of the roughening of the egg at fertilization.

(*i*) A change in the egg thus precedes membrane elevation and makes it probable that the latter process is not the primary event in the fertilization reaction.

(*j*) After membrane elevation by such artificial agents as were tested there is no increased viscosity in the egg but the cytoplasm is of the same or of less viscosity than the unfertilized egg, until cytolysis occurs.

(*k*) Cytolysis consists of an irreversible coagulation of the egg cytoplasm and appears to be due to the fact that the egg after the action of cytolytic agents is left without a resistant surface. It consequently ruptures and this injury leads to coagulation. Probably cytolysis bears no relation to activation.

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